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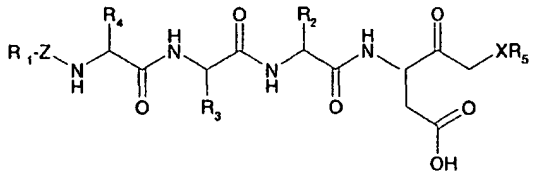

(I)

(57) Abstract

The present invention is related to the novel compounds of formula (I), their pharmaceutical compositions, and to the novel inhibition of caspases for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death.

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Caspases and Apoptosis

FIELD OF THE INVENTION

The present invention is to the discovery of a new method to block excessive or inappropriate apoptosis in a mammal.

BACKGROUND

It has been recognized for over a century that there are different forms of cell death. One form of cell death, necrosis, is usually the result of severe trauma and is a process that involves loss of membrane integrity and uncontrolled release of cellular contents, often giving rise to inflammatory responses. In contrast, apoptosis is a more physiological process that occurs in a controlled manner and is generally non-inflammatory in nature. For this reason apoptosis is often referred to as programmed cell death. The name itself (apoptosis: Greek for "dropping off", for example leaves from trees) implies a cell death that is part of a normal physiological process (Kerr et al., Br. J. Cancer, 26: 239-257 (1972)).

Apoptosis appears to be a carefully controlled series of cellular events which ultimately leads to death of the cell. This process for elimination of unwanted cells is active and requires expenditure of cellular energy. The morphological characteristics of apoptosis include cell shrinkage and loss of cell-cell contact, condensation of nuclear chromatin followed by fragmentation, the appearance of membrane ruffling, membrane blebbing and apoptotic bodies. At the end of the process, neighboring cells and macrophages phagocytose the fragments from the apoptotic cell. The process can be very fast, occurring in as little as a few hours (Bright et al., Biosci. Rep., 14: 67-82 (1994)).

The best defined biochemical event of apoptosis involves the orderly destruction of nuclear DNA. Signals for apoptosis promote the activation of specific calcium- and magnesium-dependent endonucleoases that cleave the double stranded DNA at linker regions between nucleosomes. This results in production of DNA fragments that are multiples of 180-200 base pair fragments (Bergamaschi et al., Haematologica, 79: 86-93 (1994); Stewart, JNCI, 86: 1286-1296 (1994)). When examined by agarose gel electrophoresis, these multiple fragments form a ladder pattern that is characteristic for most cells undergoing apoptosis.

There are numerous stimuli that can signal cells to initiate or promote cellular apoptosis, and these can be different in different cells. These stimuli can include glucocorticoids, TNFa, growth factor deprivation, some viral proteins, radiation and anticancer drugs. Some of these stimuli can induce their signals through a variety of cell surface receptors, such as the TNF / nerve growth factor family of receptors, which include CD40 and Fas/Apo-1 (Bright et al., supra). Given this diversity in stimuli that cause apoptosis it has been difficult to map out the signal transduction pathways and molecular factors involved in apoptosis. However, there is evidence for specific molecules being involved in apoptosis.

The best evidence for specific molecules that are essential for apoptosis comes from the study of the nematode C. elegans. In this system, genes that appear to be required for induction of apoptosis are Ced-3 and Ced-4. These genes must function in the dying cells and, if either gene is inactivated by mutation, cell death fails to occur (Yuan et al., Devel. Biol., 138: 33-41 (1990)). In mammals, genes that have been linked with induction of apoptosis include the proto-oncogene c-myc and the tumor suppresser gene p53 (Bright et al., supra; Symonds et al., Cell, 78: 703-711 (1994)).

In this critical determination of whether or not to undergo apoptosis, it is not surprising that these are genes that program for proteins that inhibit apoptosis. An example in C. elegans is Ced-9. When it is abnormally activated, cells survive that would normally die and, conversely, when Ced-9 is inactivated cells die that would normally live (Stewart, B.W., supra). A mammalian counterpart is bcl-2, which had been identified as a cancer-causing oncogene. This gene inhibits apoptosis when its product is overexpressed in a variety of mammalian cells, rendering them less sensitive to radiation, cytotoxic drugs and apoptotic signals such as c-myc (Bright et al., supra). Some virus protein have taken advantage of this ability of specific proteins to block apoptosis by producing homologous viral proteins with analogous functions. An example of such a situation is a protein produced by the Epstein Barr virus that is similar to bcl-2, which prevents cell death and thus enhances viral production (Wells et al., J. Reprod. Fertil., 101: 385-391 (1994)). In contrast, some proteins may bind to and inhibit the function of bcl-2 protein, an example being the protein bax (Stewart, B.W., supra). The overall picture that has developed is that entry into apoptosis is regulated by a careful balancing act between specific gene products that promote or inhibit apoptosis (Barinaga, Science, 263: 754-756 (1994).

Apoptosis is an important part of normal physiology. The two most often sited examples of this are fetal development and immune cell development. In development of

the fetal nervous system, over half of the neurons that exist in the early fetus are lost by apoptosis during development to form the mature brain (Bergamaschi et al., Haematologica, 79: 86-93 (1994)). In the production of immune competent T cells (and to a lesser extent evidence exists for B cells), a selection process occurs that eliminates cells that recognize and react against self. This selection process is thought to occur in an apoptotic manner within areas of immune cell maturation (Williams, G. T., J. Pathol., 173: 1-4 (1994); Krammer et al., Curr. Opin. Immunol., 6: 279-289 (1994)).

Dysregulation of apoptosis can play an important role in disease states, and diseases can be caused by both excessive or too little apoptosis occurring. An example of diseases associated with too little apoptosis would be certain cancers. There is a follicular B-cell lymphoma associated with an aberrant expression of functional bcl-2 and an inhibition of apoptosis in that cell (Bergamaschi et al., supra). There are numerous reports that associate deletion or mutation of p53 with the inhibition of apoptosis and the production of cancerous cells (Kerr et al., Cancer, 73: 2013-2026 (1994); Ashwell et al., Immunol. Today, 15: 147-151, (1994)). In contrast, one example of excessive or inappropriate apoptosis is the loss of neuronal cells that occurs in Alzheimer disease, possible induced by b-amyloid peptides (Barr et al., BioTechnology, 12: 487-493 (1994)). Another example of excessive or inappropriate apoptosis is the loss of neurons and oligondendroglia that occur in traumatic spinal cord injury ((Springer er al) Nature Medicine 5: 943-946 1999)). Other examples include excessive apoptosis of $CD4^+$ T cells that occurs in HIV infection, of cardiac myocytes during infarction / reperfusion and of neuronal cells during ischemia (Bergamaschi et al., supra); Barr et al., supra).

Some pharmacological agents attempt to counteract the lack of apoptosis that is observed in cancers. Examples include topoisomerase II inhibitors, such as the epipodophyllotoxins, and antimetabolites, such as ara-c, which have been reported to enhance apoptosis in cancer cells (Ashwell et al., supra). In many cases with these anti-cancer drugs, the exact mechanism for the induction of apoptosis remains to be elucidated.

In the last few years, evidence has built that ICE and proteins homologous to ICE (Caspases) play a key role in apoptosis. This area of research has been spurred by the observation of homology between the protein coded by Ced-3, a gene known to be critical for C. Elegans apoptosis, and ICE (Caspase 1). These two proteins share 29% amino acid identity, and complete identity in the 5 amino acid portion thought to be responsible for protease activity (QACRG) (Yuan et al., Cell, 75: 641-652 (1993)). Additional homologies are observed between ICE and the product of the nedd-2 gene in mice, a gene

suspected of involvement in apoptosis in the developing brain (Kumar et al., Genes Dev., 8: 1613-1626 (1994)) and Ich-1 (Caspase 2) and CPP32 (Caspase 3), human counterparts of nedd-2 isolated from human brain cDNA libraries (Wang et al., Cell, 78: 739-750 (1994); Fernandes-Alnemiri et al., J. Biol. Chem., 269: 30761-30764 (1994)).

Further proof for the role of these proteins in apoptosis comes from transfection studies. Over expression of murine ICE caused fibroblasts to undergo programmed cell death in a transient transfection assay (Miura et al., Cell, 75: 653-660 (1993)). Cell death could be prevented by point mutations in the transfected gene in the region of greatest homology between ICE and Ced-3. As very strong support for the role of ICE in apoptosis, the authors showed that ICE transfection-induced apoptosis could be antagonized by overexpression of bcl-2, the mammalian oncogene that can prevent programmed cell death (Miura et al., supra). Additional experiments were performed using the crmA gene. This gene of the cowpox virus encodes a serpin protein, a family of proteins that are inhibitors of proteases (Ray et al., Cell, 69: 597-604 (1992)). Specifically, the protein of crmA has been shown to inhibit processing of pro-interleukin -1b by ICE. (Gagliardini et al. Science, 263: 826-828 (1994)) showed that microinjection of the crmA gene into dorsal root ganglion neurons prevented cell death induced by nerve growth factor deprivation. This result shows that ICE is involved in neuronal cell apoptosis. A more direct demonstration of ICE involvement comes from experiments in which ICE transfection is coupled with the co-expression of crmA, demonstrating a crmA-induced suppression of the ICE-induced apoptosis response (Miura et al., supra; Wang et al., supra).

In addition to ICE, researchers have examined the ability of Caspase genes to promote apoptosis. (Kumar et al. supra) demonstrated that over expression of nedd-2 in fibroblasts and neuroblastoma cells resulted in cell death by apoptosis and that this apoptosis could also be suppressed by expression of the bcl-2 gene. Most recently, Wang et al., (Wang et al. , supra) examined the over expression of Ich-1 in a number of mammalian cells. Expression resulted in cell apoptosis, which could be antagonized by bcl-2 co-expression. Mutation of a cysteine residue, contained within the QACRG motif and presumed to be critical for protease function, to serine abolished apoptotic activity.

Further evidence for a role of a cysteine protease in apoptosis comes from a recent report by Lazebnik et al. (Nature, 371: 346-347 (1994)). These authors have used a cell-free system to mimic and study apoptosis. In their system there is a protease activity that cleaves the enzyme poly(ADP-ribose) polymerase at a site identical to a cleavage site in pre-interleukin-1b. However, this yet to be isolated protease and ICE appear to be

different and to act on different substrate proteins. Blockade of protease activity in the system, using non-selective cysteine protease inhibitors, resulted in inhibition of apoptosis.

Taken together, the above evidence provides striking involvement of Caspases in the induction of apoptosis in mammalian cells. Brain interleukin-1 has been reported to be elevated in Alzheimer disease and Down syndrome (Griffin et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7611-7615 (1989)). There are also reports that interleukin-1 can increase the mRNA and production of b-amyloid protein, a major component of senile plaques in Alzheimer disease as well as in brains of people with Down syndrome and with aging (Forloni et al., Mol. Brain Res., 16: 128-134 (1992); Buxbaum et al., Proc. Natl. Acad. Sci. U. S. A., 89: 10075-10078 (1992); Goldgaber et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7606-7610 (1989)). These reports can be viewed as additional evidence for the involvement of ICE in these diseases and the need for use of a novel therapeutic agent and therapy thereby.

To date, no useful therapeutic strategies have blocked excessive or inappropriate apoptosis. In one patent application, EPO 0 533 226 a novel peptide structure is disclosed which is said to be useful for determining the activity of ICE, and therefore useful in the diagnoses and monitoring of IL-1 mediated diseases. Therefore, a need exists to find better therapeutic agents which have non-toxic pharmacological and toxicological profiles for use in mammals. These compounds should block excessive or inappropriate apoptosis cells, and hence provide treatment for diseases and conditions in which this condition appears.

SUMMARY OF THE INVENTION

The present invention is to the novel compounds of Formula (I), their pharmaceutical compositions, and to the novel inhibition of caspases for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death. The compounds of Formula I are most effective in inhibiting caspases three, seven and eight.

Another aspect of the present invention is to a pharmaceutical composition comprising a compound of Formula (I), or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier or diluent.

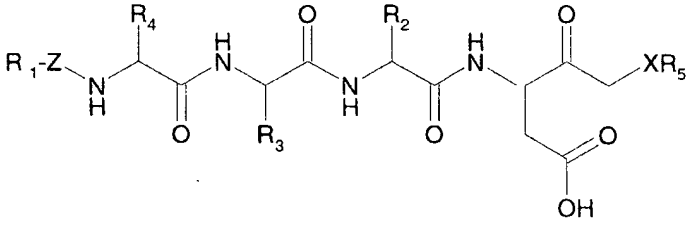
Another aspect of the present invention is to a method for the treatment of diseases or disorders associated with excessive IL-1b convertase activity, in a mammal in

need thereof, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

Another aspect of the present invention is to a method of preventing or reducing apoptosis in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

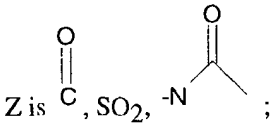
Another aspect of the present invention is to a method of blocking or decreasing the production of IL-1b and/or TNF, in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

The compounds of Formula I are represented by the structure



(I)

wherein

Z is  ;

R_1 is alkyl, alkylaryl or aryl;

R_2, R_3, and R_4 are selected from naturally occuring amino acids;

X is sulfur, oxygen or nitrogen and when X is sulfur, R_5 is C_{1-6} alkyl, alkylaryl or aryl

and when X is nitrogen R_5 is Y-R_6 and Y is SO_2, or  ;

The preferred compounds of formula I are when

R_1 is alkyl, alkylaryl or aryl;

R_2 is valine, isoleucine or threonine;

R_3 is glutamic acid or valine;

R_4 is aspartic acid;

R_5 is $Y—R_6$ and Y is SO_2, or $\overset{O}{\overset{\|}{C}}$;

and X is Nitrogen.

Compounds exemplified by Formula (I) include, but are not limited to:

Acetyl-aspartylglutamylvalylaspartyl benzoylaminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropionyl)aminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid phenylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropyl)thiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid phenylthiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylthiomethylketone

The most preferred compounds are:
Acetyl-aspartylglutamylvalylaspartyl benzoylaminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropionyl)aminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid phenylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylthiomethylketone.

The term "excessive IL-1b convertase activity" is used herein to mean an excessive expression of the protein, or activation of the enzyme.

The term "C_{1-6} alkyl" or "alkyl" is used herein to mean both straight and branched chain radicals of 1 to 6 carbon atoms, unless the chain length is otherwise specified, including, but not limited to, methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *sec*-butyl, *iso*-butyl, *tert*-butyl, and the like.

The term "heteroaryl" (on its own or in any combination, such as "heteroaryloxy", or "heteroaryl alkyl") is used herein to mean a 5-10 membered aromatic ring system in which one or more rings contain one or more heteroatoms selected from the group consisting of N, O or S, such as, but not limited, to pyrrole, pyrazole, furan, thiophene, quinoline, isoquinoline, quinazolinyl, pyridine, pyrimidine, oxazole, oxadiazole, tetrazole, thiazole, thiadiazole, triazole, imidazole, benzimidazole, benzothiaphene, benzopyrrole, or benzofuran.

The term "aryl" (on its own or in any combination, such as "aryloxy", or "arylalkyl") is used herein to mean a phenyl and naphthyl ring.

The term "cycloalkyl" is used herein to mean cyclic radicals, preferably of 3 to 7 carbons, including but not limited to cyclopropyl, cyclopentyl, cyclohexyl, and the like.

The term "halo" or "halogens", is used herein to include, unless otherwise specified, chloro, fluoro, bromo and iodo.

The present invention contains the inhibition of caspases by compounds of Formula (I). What is meant by the term "caspases" are fragment, homologs, analogs and derivatives of the polypeptides Interleukin-1 b converting enzyme (or convertase). These analogs are structurally related to the caspase family. They generally encode a protein (s) which exhibits high homology to the human ICE over the entire sequence. Preferably, the pentapeptide QACRG is conserved. The caspases , which may include many natural allelic variants (such as substitutions, deletion or addition of nucleotides) does not substantially alter the function of the encoded polypeptide. That is they retain essentially the same biological function or activity as the ICE protease, although it is recognized that the biological function may be enhanced or reduced activity. The suitable activity is not IL-1b convertase activity, but the ability to induce apoptosis or involved in programmed cell death in some manner. Suitable caspases encompasses within this invention are those described in PCT US94/07127 filed 23 June 1994, Attorney Docket No.: 325800-184; and in USSN 08/334,251, filed 1 November 1994, Attorney Docket No.: 325800-249 whose disclosures are incorporated herein by reference in their entirety.

The term "blocking or inhibiting, or decreasing the production of IL-1b and/or TNF" as used herein refers to:

a) a decrease of excessive levels, or a down regulation, of the cytokine in a human to normal or sub-normal levels by inhibition of the *in vivo* release of the cytokine; or

b) a down regulation, at the genomic level, of excessive *in vivo* levels of the cytokine (IL-1 or TNF) in a human to normal or sub-normal levels; or

c) a down regulation, by inhibition of the direct synthesis of the cytokine (IL-1, or TNF) as a postranslational event; or

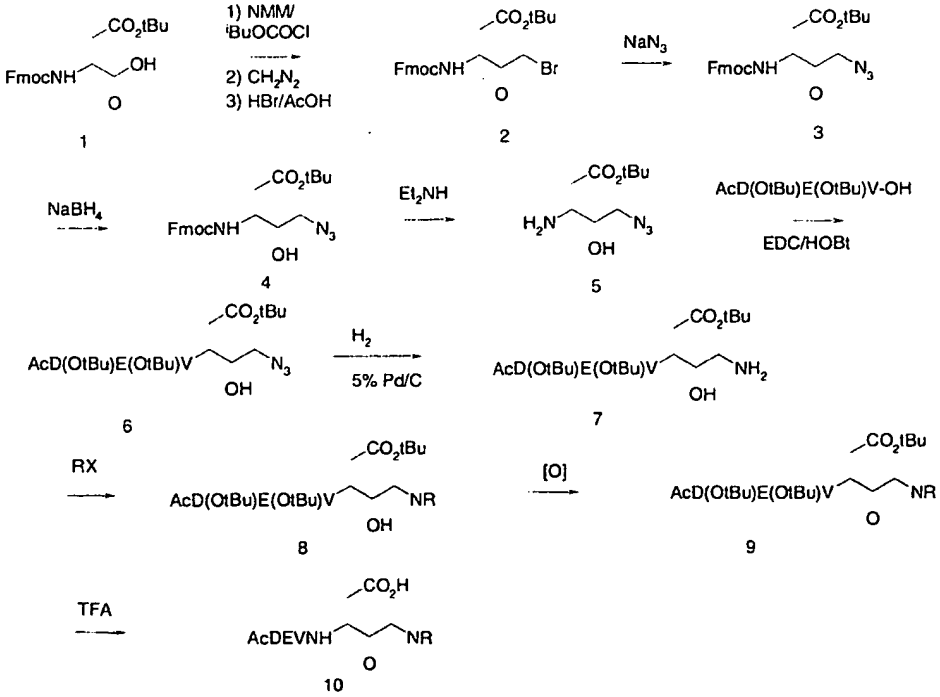
d) a down regulation, at the translational level, of excessive *in vivo* levels of the cytokine (IL-1, or TNF) in a human to normal or sub-normal levels.

The blocking or inhibiting, or decreasing the production of IL-1b and/or TNF is a discovery that the compounds of Formula (I) are inhibitors of the cytokines, IL-1 and TNF is based upon the effects of the compounds of Formulas (I) on the production of the IL-1 and TNF in *in vitro* and *in vivo* assays which are well known and recognized in the art, some of which are described herein.

Compound of the present invention may be synthesized in accordance with the schemes illustrated below.

General Scheme

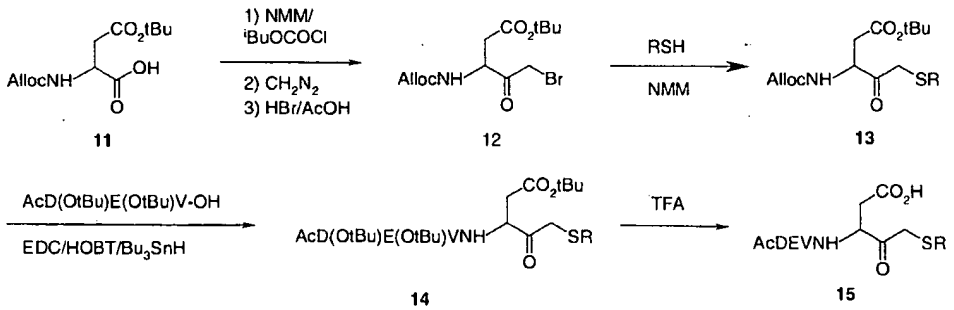
Diaminomethylketones



Scheme 1

FmocAspartic acid t-Butyl ester **1** is converted to the corresponding bromomethylketone **2** by treatment with isobutyl chlorformate and N-methylmorpholine in THF at -20 ^{0}C, followed by treatment with diazomethane, and quenching of the diazomethylketone with 48% HBr in acetic acid. The bromomethylketone is then converted to the azidomethylketone **3** with NaN_3 in polar solvent mixtures such as water/DMF or water/CH_3CN. The ketone may then be reduced using $NaBH_4$ in methanol to give the corresponding alcohol **4**. Removal of the Fmoc group can then be effected using standard Fmoc deprotection conditions to give the free amine **5**. This amine is coupled to the tripeptide AcD(OtBu)E(OtBu)V-OH (Prepared using standard Fmoc-amino acid coupling chemistry from the following monomers: FmocD(OtBu)-OH, FmocE(OtBu)-OH, V-OBn. N –terminal functionalization as an amide, sulfonamide or urea is effected under standard conditions on the appropriately protected tripeptide) using standard amino acid coupling conditions to give the tetrapeptide azidomethyl alcohol **6**. The azide is then reduced to the primary amine using reducing condition such as hydrogenation using Pd/C in methanol or PPh_3/H_2O in THF to give the corresponding amino alcohol **7**. The amine may then be reacted with an electrophile such as an acylchloride or sulfonychloride in an organic solvent such as THF or CH_2Cl_2 using a tertiary amine such as Et_3N or Hunig's base to give amides or sulfonamides **8**. This intermediate is then oxidized to the ketones **9** with an oxidizing agent such as the tetrapropylammonium perruthenate/4-methylmorpholine N-oxide, pyridine sulfur trioxide or Dess Martin reagent. The tetrapeptide derivative may then be deprotected with TFA to give the desired amidomethylketone or sulfonamidomethylketone tetrapeptide derivatives **10**.

Thiomethylketones



Scheme 2

AllocAspartic acid t-Butyl ester **11** is converted to the corresponding bromomethylketone **12** by treatment with isobutyl chlorformate and N-methylmorpholine in THF at -20 ^{0}C, followed by treatment with diazomethane, and quenching of the diazomethylketone with 48% HBr in acetic acid. The bromide is diplaced with a mercaptan and a base such as N-methylmorpholine or Hunig's base in solvents such as CH_2Cl_2, DMF, DMSO or THF to afford the thiomethyketones **13**. Coupling with AcD(OtBu)E(OtBu)V-OH is carried out with an in situ deprotection of the alloc group in the presence of EDC/HOBT (Chapman, K. T. *Bioorg. Med. Chem. Lett.* **2**, 613, 1992) to give the tetrapeptides **14**. Finally, deprotection with TFA yields the tetrapeptide thiomethylketones **15**.

Example 1 (SB255427)

Acetyl-aspartylglutamylvalylaspartyl benzoylaminomethylketone

1a) Boc-valine benzyl ester

To Boc-valine (12 g, 55.3 mmol) and benzyl alcohol (5.67g, 52.5 mmol) in 150 mL of CH_2Cl_2 at 0 ^{0}C was added DMAP (7.0 g, 58.1 mmol), and the solution was stirred 10 min. The resulting solution was treated with EDC (11.1 g, 58.1 mmol) and stirred overnight at room temperature. The solution was washed with 100 mL of 3N HCl. The aqueous layer was extracted twice with 50 mL of CH_2Cl_2 and the combined organic layers were dried over magnesium sulfate, filtered and concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with 10-25% EtOAc/Hexanes to give the title compound as a clear colorless oil (14.5 g, 90%). ES (+) MS m/e = 330 (MNa^+).

1b) Valine benzyl ester

To a solution of Boc-valine benzyl ester (14.3 g, 46.4 mmol) in 100 mL CH_2Cl_2 at 0 ^{0}C was added 100 mL of trifluoroacetic acid dropwise over 5 min. The solution was warmed to room temperature and stirred 1 h. The solution was concentrated under reduced pressure to a small volume and purified by silica gel chromatography with 5-10% CH_3OH/CH_2Cl_2 to give an oil. The oil was then dissolved in 100 mL of CH_2Cl_2 and washed with 100 mL of 1N NaOH. The aqueous layer was extracted with CH_2Cl_2 (3X100 mL). The combined organic layers were dried over magnesium sulfate, filtered and

concentrated under reduced pressure to give the title compound as an oil (8.2 g, 85%). ES (+) MS m/e = 208 (MH^+).

1c) Fmoc-glutamyl(t-Bu)valine benzyl ester

To a solution of valine benzyl ester (8.2 g, 39.8 mmol), Fmoc-glutamic(t-Bu) acid (17.7 g, 39.8 mmol), and HOBT (5.37, 39.8 mmol) in 100 mL of CH_2Cl_2 at 0 ^0C was added EDC (8.0 g, 41.8 mmol), and the resulting solution was stirred at room temperature overnight. The solution was washed with 100 mL of 3N HCl and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over magnesium sulfate, filtered and concentrated under reduced pressure to give a solid. The solid was purified by silica gel chromatography with 75% EtOAc/Hexanes to give the title compound as white solid (23.6 g, 97%). ES (+) MS m/e = 615 (MH^+).

1d) Glutamyl(t-Bu)valine benzyl ester

To a solution of Fmoc-glutamyl(t-Bu)valine benzyl ester (23.6g, 38.4 mmol) in 150 mL of CH_2Cl_2 was added 40 mL of diethylamine, and the resulting solution was stirred for 4.5 h. The solution was concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with 50% EtOAc/Hexanes, followed by 5% CH_3OH/CH_2Cl_2 to give the title compound as a colorless oil (14.5g, 96%). ES (+) MS m/e = 393 (MH^+).

1e) Fmoc-aspartyl(t-Bu)glutamyl(t-Bu)valyl benzyl ester

Preparation according to the procedure of example 1c) except using Fmoc-aspartic(t-Bu) acid and glutamyl(t-Bu)valine benzyl ester instead of valine-benzyl ester and Fmoc-glutamic(t-Bu) acid respectively afforded the title compound as a white foam in 79% yield. ES (+) MS m/e = 808 (MNa^+).

1f) Aspartyl(t-Bu)glutamyl(t-Bu)valyl benzyl ester

Preparation according to the procedure of example 1d) except using Fmoc-aspartyl(t-Bu)glutamyl(t-Bu)valine benzyl ester instead of Fmoc-glutamyl(t-Bu)valine benzyl ester afforded the title compound as an oil in 65% yield. ES (+) MS m/e = 563 (MH^+).

1g) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valine

To aspartyl(t-Bu)glutamyl(t-Bu)valyl benzyl ester (9.0 g, 16 mmol) was added 200 mL of 10% 5:1 pyridine/acetic anhydride in CH_2Cl_2, and the resulting solution was stirred 4 h. The solution was washed with 100 mL of 3N HCl, 100 mL of 10% potassium carbonate, and 100 mL of water. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give a white solid. The crude material was purified by silica gel chromatography with 3-10% CH_3OH/CH_2Cl_2 to give the title compound as a white solid.

A mixture of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valyl benzyl ester (5g) and palladium black (100 mg) was pressurized to 50 psi with hydrogen on a Parr shaker for 3 h. The catalyst was filtered, and removal of solvent under reduced pressure yielded the title compound as a white solid. ES (+) MS m/e = 516 (MH^+).

1h) Fmoc-aspartic(t-Bu) acid bromomethylketone

To a solution of Fmoc-aspartic(t-Bu) acid (4.1 g, 10 mmol) in 50 mL of THF at -20 ^0C was added 4-methylmorpholine (1.65 mL, 15 mmol), and the solution was stirred for 5 min. To the resulting solution was added isobutylchloroformate (1.56 mL, 12 mmol), and the mixture was stirred for 10 min. The mixture was treated with diazomethane (2.6 g, 17.7 mmol) in 24 mL of diethyl ether (generated from 1-methyl-3-nitro-1-nitrosoguanidine), and the mixture was stirred for 15 min, followed by warming to RT. The solution was stirred for 1 h at RT. The mixture was cooled to 0 ^0C, and treated with 12 mL of 2:1 48% hydrobromic acid:acetic acid dropwise. The solution was warmed to RT and stirred for 30 min. Diethyl ether was added to the solution and it was washed with 100 mL of water, 100 mL of sodium bicarbonate (sat.), and 100 mL of brine. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give the title compound as an oil which was used without further purification (5.3 g, >100%). ES (+) MS m/e = 510 (MNa^+).

1i) Fmoc-Aspartic(t-Bu) acid azidomethylketone

To a solution of Fmoc-aspartic(t-Bu) acid bromomethylketone (10 mmol) in 50 mL of acetonitrile and 7 mL of DMF at 0 ^0C was added a solution of sodium azide (1.3 g, 20 mmol) in 5 mL of water, and the mixture was warmed to room temperature. A precipitate formed and another 25 mL of DMF and 35 mL of water were added. The solution turned a clear light yellow color after stirring for 30 min, and stirring was continued for an

additional 1.5 h. The solution was concentrated under reduced pressure to remove acetonitrile, and 150 mL of EtOAc were added along with 50 mL of water. The layers were separated, and the aqueous portion was extracted twice with 50 mL of EtOAc. The combined organic layers were washed twice with 50 mL of brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure to give the title compound as a yellow oil which was used without further purification (4.5 g, 100%). ES (+) MS m/e = 473 (MNa^+).

1j) Fmoc-aspartic(t-Bu) acid azidomethylalcohol

To Fmoc-aspartic(t-Bu) acid azidomethylketone (3.5 g, 7.7 mmol) in 35 mL of methanol at 0 ^0C was added sodium borohydride (0.35 g, 9.3 mmol), and the solution was stirred for 20 min. The reaction was quenched carefully with 70 mL of 3N HCl and extracted twice with 50 mL of CH_2Cl_2. The combined organic layers were dried over magnesium sulfate, filtered and concentrated under reduced pressure to give a white solid. The solid was recrystallized from ethyl acetate/hexanes to give the title compound as a white solid (2.5 g, 71%). ES (+) MS m/e = 475 (MNa^+).

1k) Aspartic(t-Bu) acid azidomethylalcohol

Preparation according to the procedure of example 1d) except using Fmoc-aspartic acid(t-Bu) azidomethylalcohol instead of Fmoc-aspartyl(t-Bu)valine benzyl ester afforded the title compound as a light yellow solid in 86% yield. ES (+) MS m/e = 231 (MH^+).

1l) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid azidomethylalcohol

To a mixture of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valine (1.1 g, 2.2 mmol), aspartic(t-Bu) acid azidomethylalcohol (0.50 g, 2.2 mmol), and HOBT (0.29 g, 2.2 mmol) in 10 mL of CH_2Cl_2 was added EDC (0.46 g, 2.4 mmol), followed by 0.5 mL of DMF. The resulting solution was stirred overnight. To the solution was added 50 mL of CH_2Cl_2, which was then washed with 50 mL of 3N HCl, 50 mL of 10% potassium carbonate, and 50 mL of water. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give the title compound as a white solid (1.4 g, 89%). ES (+) MS m/e = 728 (MH^+).

1m) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid aminomethylalcohol

To a solution acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid azidomethylalcohol (1.2 g, 1.7 mmol) in 20 mL of methanol was added 5% Pd on carbon (0.41 g) and the resulting mixture was stirred under a balloon of hydrogen for 2.5 h. The mixture was filtered, and the solution concentrated under reduced pressure to give the title comound as an oil (1.15 g, 99%). ES (+) MS m/e = 701 (MH^+).

1n) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylalcohol

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid aminomethylalcohol (0.22 g, 0.31 mmol) in 10 mL of CH_2Cl_2 and 0.5 mL of DMF was added pyridine (0.076 mL, 0.94 mmol) followed by benzoyl chloride (0.044 mL, 0.38 mmol). The solution was stirred for 3 h, and 20 mL of CH_2Cl_2 was added. The solution was washed with 20 mL of 3N HCl and 20 mL of water. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give a solid. The solid was purified by silica gel chromatography with 5-9% CH_3OH/CH_2Cl_2 to give the title compound as a white solid (0.18 g, 80%). ES (+) MS m/e = 806 (MH^+).

1o) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylalcohol (0.16 g, 0.2 mmol) and 4-methylmorpholine N-oxide (0.035 g, 0.3 mmol) in 3 mL of CH_2Cl_2 with 4A molecular sieves was added tetrapropylammonium perruthenate (0.018 g, 0.051 mmol), and the mixture was stirred for 3 h. The solution was treated with 20 mL of CH_2Cl_2 and was then washed with 20 ml of 20% sodium sulphite, 20 mL of brine, and 20 mL of copper (III) sulfate. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with 3-7% CH_3OH/CH_2Cl_2 to give the title compound as an oil (0.039 g, 24%). ES (+) MS m/e = 804 (MH^+).

1p) Acetyl-aspartylglutamylvalylaspartic acid benzoylaminomethylketone

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone (0.039 g, 0.049 mmol) in 5 mL of CH_2Cl_2 was added 5 mL of trifluoroacetic acid. The solution was warmed to room temperature and stirred for 1 h. The

solvent was removed under reduced pressure, and the resulting residue was dissoved in CH_2Cl_2 and treated with toluene. The solvent was again removed under reduced pressure and this was repeated two more times. The residue was dissolved in a minimal amount of CH_2Cl_2 and precipitated with diethyl ether to give the title compound as a white solid (0.020 g, 65%). ES (+) MS m/e = 636 (MH^+).

Example 2 (SB255428)

Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropionyl)aminomethylketone

2a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropionoyl)aminomethylalcohol

Preparation according to the procedure of example 1n) except using hydrocinnamoyl chloride instead of benzoyl chloride afforded the title compound as an oil in 75% yield. ES (+) MS m/e = 834 (MH^+).

2b) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropionoyl)aminomethylketone

Preparation according to the procedure of example 1o) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropionoyl)aminomethylalcohol instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylalcohol afforded the title compound as an oil in 34% yield. ES (+) MS m/e = 832 (MH^+).

2c) Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropionyl)aminomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropionoyl)aminomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone afforded the title compound as a white solid in 20% yield. ES (+) MS m/e = 664 (MH^+).

Example 3 (SB256047)

Acetyl-aspartylglutamylvalylaspartic acid phenylsulfonamidomethylketone

3a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylsulfonamidomethylalcohol

Preparation according to the procedure of example 1n) except using benzenesufonyl chloride instead of benzoyl chloride afforded the title compound as an oil in 24% yield. ES (+) MS m/e = 842 (MH^+).

3b) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylsulfonamidomethylketone

Preparation according to the procedure of example 1o) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylsulfonamidomethylalcohol instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylalcohol afforded the title compound as an oil in 16% yield. ES (+) MS m/e = 840 (MH^+).

3c) Acetyl-aspartylglutamylvalylaspartic acid phenylsulfonamidomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylsulfonamidomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone afforded the title compound as a white solid in 88% yield. ES (+) MS m/e = 672 (MH^+).

Example 4 (SB256354)

Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone

4a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid methylsulfonamidomethylalcohol

Preparation according to the procedure of example 1n) except using methanesulfonyl chloride instead of benzoyl chloride afforded the title compound as an oil in 22% yield. ES (+) MS m/e = 780 (MH^+).

4b) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid methylsulfonamidomethylketone

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid methylsulfonamidomethylalcohol (0.056 g, 0.072 mmol) and triethylamine (0.12 mL, 0.86 mmol) in 0.5 mL of DMSO was added pyridine sulfur trioxide at 40 ^{0}C. The solution was stirred for 1.5 h. Ethyl acetate was added to the solution, and the organic layer was washed with 25 mL of 3N HCl, 25 mL of sodium bicarbonate (sat.), 25 mL of water, and 25 mL of brine. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to give the title compound as an oil in 27% yield. ES (+) MS m/e = 778 (MH^+).

4c) Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid_methylsulfonamidomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone afforded the title compound as a white solid in 58% yield. ES (-) MS m/e = 608 (MH-).

Example 5 (SB262481)

Acetyl-aspartylglutamylvalylaspartic acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone

5a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylalcohol

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid aminomethylalcohol (50 mg, 70 umol) in 500 uL of pyridine was added (5-acetamido-3-methylthiophene)-2-sulfonyl chloride (22 mg, 87 umol). The solution was stirred for 18 h. 20 mL of CH_2Cl_2 was added, and the solution was washed with 20 mL of 3N HCl. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The solid was purified by silica gel chromatography with 2-5% CH_3OH/CH_2Cl_2 to give the title compound (38 mg). ES (+) MS m/e = 920 (MH^+).

5b) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylalcohol (20 mg, 22 umol) in 2:1 CH_2Cl_2:CH_3CN (1.5 mL) was added Dess-Martin oxidant (40 mg). The solution was stirred for 2 h, and an additonal 2 mL of CH_2Cl_2 and 50 mg of oxidant was added. Solutions of 20% sodium metasulfate (5 mL) and saturated sodium bicarbonate (10 mL) were added, and the mixture was extracted with CH_2Cl_2. The organic layer was dried over magnesium sulfate, filtered, concentrated under reduced pressure, and silica gel flash chromatography (3-10% CH_3OH/CH_2Cl_2) afforded the title compound (18 mg). ES (+) MS m/e = 918 (MH^+).

5c) Acetyl-aspartylglutamylvalylaspartic acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone afforded the title compound in quantitative yield. ES (-) MS m/e = 748 (MH-).

Example 6 (SB262482)

Acetyl-aspartylglutamylvalylaspartic acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone

6a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylalcohol

Preparation according to the procedure of example 5a) except using (5-benzoylaminomethylthiophene)-2-sulfonyl chloride instead of (5-acetamido-3-methylthiophene)-2-sulfonyl chloride afforded the title compound. ES (+) MS m/e = 981 (MH^+).

6b) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone

Preparation according to the procedure of example 5b) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylalcohol instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylalcohol and using CH_2Cl_2 as solvent afforded the title compound. ES (+) MS m/e = 979 (MH^+).

6c) Acetyl-aspartylglutamylvalylaspartic acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone afforded the title compound in quantitative yield. ES (-) MS m/e = 809 (MH-).

Example 7 (SB-254510)

Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropyl)thiomethylketone

7a) Alloc-aspartic(t-Bu) acid bromomethylketone

To a solution of 2.59 g (9.9 mmol) of Alloc-aspartic(t-Bu) acid (prepared from aspartic(t-Bu) acid and Alloc-Cl under Schotten-Baumann conditions) and 4-methylmorpholine (1.2 mL) in THF (50 mL) was added isobutylchloroformate (1.42 mL) dropwise at -20 ^{0}C. The solution was stirred for 30 min, and a solution of ethereal diazomethane (20 mmoL) was added at 0 ^{0}C. The solution was stirred for 30 min, and a solution of 2:1 48% hydrobromic acid:acetic acid (10 mL) was added dropwise at 0 ^{0}C. The solution was washed with water, and poured slowly into an ice cold solution of saturated sodium bicarbonate. The organic layer was separated, washed with brine, and dried over magnesium sulfate. Removal of solvent under reduced pressure and silica gel flash chromatography afforded the title compound as an oil. ^{1}H NMR (250 mHz, $CDCl_3$) δ1.45 (s, 3H), 2.76 (dd, J=17 Hz, J=5.9 Hz, 1H), 2.98 (dd, J=17 Hz, J=5.9 Hz, 1H), 4.20 (s, 2H), 4.62 (d, J=6.6 Hz, 2 H), 4.75 (m, 1H), 5.2-5.4 (m, 2H), 5.8-6.0 (m, 2H).

7b) Alloc-aspartic(t-Bu) acid (3-phenylpropyl)thiomethylketone

To a solution of Alloc-aspartic(t-Bu) acid bromomethylketone (206 mg, 0.61 mmol) in CH_2Cl_2 (5 mL) was added 3-phenylpropyl mercaptan (102 uL, 0.67 mmol) and diisopropylethylamine (117 uL, 0.67 mmol). The solution was stirred for 1 h at RT. The mixture was washed with 3N HCl, the organic extract was dried over magnesium sulfate, and the solvent was removed under reduced pressure. Silica gel flash chromatography (7.5-10% ethyl acetate/hexanes) yielded the title compound (197 mg, 77%). ^1H NMR (250 mHz, $CDCl_3$) δ1.44 (s, 3H), 1.90 (m, 2H), 2.51 (t, J=7.6 Hz, 2H), 2.70 (t, J=7.2 Hz, 2H), 2.76 (dd, J=17 Hz, J=5.9 Hz, 1H), 2.93 (dd, J=17 Hz, J=5.9 Hz, 1H), 3.43 (m, 2H), 4.59 (d, J=6.6 Hz, 2 H), 4.77 (m, 1H), 5.2-5.4 (m, 2H), 5.8-6.0 (m, 2H). 7.0-7.4 (m, 5H).

7c) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropyl)thiomethylketone

To a solution of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (273 mg, 0.53 mmol) and Alloc-aspartic(t-Bu) acid (3-phenylpropyl)thiomethylketone (197 mg, 0.48 mmol) in 1:1 DMF:CH_2Cl_2 (3 mL) was added dichlorodiphenylpalladium (61 mg) at 0 ^0C. EDC (139 mg, 0.96 mmol) and HOBT (130 mg, 0.96 mmol) were added, followed by the addition of tributyltin hydride (650 uL, 2.4 mmol) dropwise. The solution was stirred at RT for 24 h.. Water (10 mL) was added, and the mixture was extracted with CH_2Cl_2. The organic extracts were dried over magnesium sulfate, the solvent was removed under reduced pressure and the residue was purified using silica gel flash chromatography (30-75% ethyl acetate/hexanes) to yield the title compound (160 mg, 40%). ES (+) MS m/e = 835 (MH^+).

7d) Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropyl)thiomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid (3-phenylpropyl)thiomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone, and adding 1% anisole afforded the title compound in quantitative yield. ES (-) MS m/e = 665 (MH-).

Example 8 (SB-255198)

Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone

8a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid cyclohexylthiomethylketone

Preparation according to the procedures of examples 7b) and 7c) except using cyclohexyl mercaptan instead of 3-phenylpropyl mercptan afforded the title compound. ES (+) MS m/e = 799 (MH^+).

8b) Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid cyclohexylthiomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone, and adding 1% anisole afforded the title compound. ES (-) MS m/e = 629 (MH-).

Example 9 (SB-255199)

Acetyl-aspartylglutamylvalylaspartic acid phenylthiomethylketone

9a) Acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylthiomethylketone

Preparation according to the procedures of examples 7b) and 7c) except using phenyl mercaptan instead of 3-phenylpropyl mercptan afforded the title compound. ES (+) MS m/e = 793 (MH^+).

9b) Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone

Preparation according to the procedure of example 1p) except using acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid phenylthiomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone, and adding 1% anisole afforded the title compound. ES (-) MS m/e = 623 (MH-).

Example 10 (SB-255199)

Acetyl-aspartylglutamylvalylaspartic acid methylthiomethylketone

10a) Alloc-aspartic(t-Bu) acid methylthiomethylketone

Preparation according to the procedures of example 7b) except using sodium methylmercaptide for 3-phenylpropyl mercaptan and running the reaction in the absence of added amine base, and example 7c) except using Alloc-aspartic(t-Bu) acid methylthiomethylketone instead of Alloc-aspartic(t-Bu) acid (3-phenylpropyl)thiomethylketone afforded the title compound. ES (+) MS m/e = 731 (MH^+).

10b) Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone

Preparation according to the procedure of example 1p) except using Alloc-aspartic(t-Bu) acid methylthiomethylketone instead of acetyl-aspartyl(t-Bu)glutamyl(t-Bu)valylaspartic(t-Bu) acid benzoylaminomethylketone, and adding 1% anisole afforded the title compound. ES (-) MS m/e = 561 [$(M-H)^-$]

Preparation of Active Caspase 3

Full length Caspase 3 was expressed intracellularly in E.coli with N-terminal hexa His tag. E coli cells were lysed in 10 ml/g of cells of lysis buffer (50 mM Na phosphate pH 7.2, 0.1 M NaCl, 0.1 % Tween 20, and 10 mM b-mercaptoethanol) using Microfluidics M110Y homogenizer at 10,000 psi. After centrifugation, Caspase 3 activity was detected in lysate supernatant. The supernatant was buffer-exchanged on Sephadex G25 column equilibrated with 20 mM TrisHCl, 10 % Sucrose, 0.1 % CHAPS, 2 mM DTT, pH 7.8 (TSCD). Fractions containing Caspase 3 activity was applied to DEAE Toyopearl 650 M (Supelco Inc) equilibrated with Buffer TSCD. The column was eluted with a linear gradient of 20 mM to 120 mM of Tris Hcl pH 7.8 in TSCD. Caspase 3 was eluted in early of the gradient before the majority of impurities eluted. This partially purified Capase 3 was used for inhibitor screening. All operations were performed at 4°C and Caspase activity was measured using substrate, DEVD-AMC , and Dynatach Fluolite 1000 plate reader.

Caspase 3 Inhibition Assay

Caspase 3 was assayed at 30 degrees C in 96-well plates using the fluorogenic tetrapeptide substrate *N*-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-7-amido-4-

methylcoumarin (Ac-**DEVD**-AMC). The assays were conducted at pH 7.5 in a buffered system containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS, and 1-50 uM DTT. The concentration of substrate was fixed at 10 uM. Fluorescence of the liberated 7-amino-4-methylcoumarin was continuously monitored at 460 nm following excitation at 360 nm.

Compound Testing

Compounds were tested at a single dose of 50 to 100 uM. Activity was monitored as described above over a 30 to 60-minute time period following the simultaneous addition of substrate and inhibitor to enzyme to initiate the reaction. The progress curves thus generated were fit by computer to Eq. 1 in order to assess potency and/or time-dependency:

$$v = \frac{(V_o(1 - e^{-k_{obs}t})}{k_{obs}} \qquad (1)$$

Representative compounds of formula (I) have demonstrated positive inhibitory activity in the above noted assay.

Apoptosis Assay (Jurkat Cells):

Materials : Compounds

Compounds were made as stocks (5-100 mM) in dimethylsulfoxide (DMSO) and diluted in DMSO to provide final concentrations, with DMSO concentrations ranging from 0.1-1%.

Preparation of cells

Jurkat cells were obtained from American Type Culture Collection and grown in RPMI-1640 media supplemented with 10% fetal bovine serum at 37°, 5% CO_2. Cells were seeded in T-flasks at 0.03 to 0.08 x 10^6 cells / ml and used for experiments at 0.5 to 1.0 x 10^6 cells / ml. Other proliferative cells can be used with apoptosis induced by anti-fas, camptothecine, cerimide or TNF.

Apoptosis Assay

A method for measuring apoptosis is to quantitate the amount of broken DNA fragments using a fluorescent end-labeling method, a system used in the ApopTag kit from Oncor (Gaithersburg, MD). In brief, the enzyme terminal deoxynucleotidyl transferase

extends the DNA fragments with digoxigenin-containing nucleotides, which are then dected with an antidigoxigenin antibody carring fluorescein to allow dection by fluorescence (494 nm excitation and 523 nm emission). Propidium iodide is used as counter stain to measure total DNA content. Flow cytometric analysis was done on Becton-Dickinson (Rutherfor, NJ) FACScan instrument using CellQuest software.

METHODS OF TREATMENT

For therapeutic use the compounds of the present invention will generally be administered in a standard pharmaceutical composition obtained by admixture with a pharmaceutical carrier or diluent selected with regard to the intended route of administration and standard pharmaceutical practice. For example, they may be administered orally in the form of tablets containing such excipients as starch or lactose, or in capsule, ovules or lozenges either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavouring or colouring agents. They may be injected parenterally, for example, intravenously, intramuscularly or subcutaneously. For parenteral administration, they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The choice of form for administration as well as effective dosages will vary depending, inter alia, on the condition being treated. The choice of mode of administration and dosage is within the skill of the art.

The compounds of the present invention, particularly those noted herein or their pharmaceutically acceptable salts which are active when given orally, can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerin, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical

carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. Preferably the composition is in unit dose form such as a tablet or capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilized and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises a compound or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

The pharmaceutically acceptable compounds of the invention will normally be administered to a subject in a daily dosage regimen. For a patient this may be, for example, from about .001 to about 100mg/kg, preferably from about 0.001 to about 10mg/kg animal body weight. A daily dose, for a larger mammal is preferably from about 1 mg to about 1000 mg, preferably between 1 mg and 500 mg or a pharmaceutically acceptable salt thereof, calculated as the free base, the compound being administered 1 to 4 times per day. Unit dosage forms may contain from aobut 25µg to about 500mg of the compound.

There are many diseases and conditions in which dysregulation of apoptosis plays an important role. All of these conditions involve undesired, deleterious loss of specific cells with resulting pathological consequences.

Bone remodeling involves the initial resorption by osteoclasts, followed by bone formation by osteoblasts. Recently, there have been a number of reports of apoptotic events occurring during this process. Apoptotic events have been observed in both the bone forming and bone resorbing cells *in vitro* and indeed at the sites of these remodeling units *in vivo*.

Apoptosis has been suggested as one of the possible mechanisms of osteoclast disappearance from reversal sites between resorption and formation. TGF-ß1 induces apoptosis (approx. 30%) in osteoclasts of murine bone marrow cultures grown for 6 days *in vitro* . (Hughes, et al., *J. Bone Min. Res.* **9** , S138 (1994)). The anti-resorptive bisphosphonates (clodronate, pamidronate or residronate) promote apoptosis in mouse osteoclasts *in vitro* and *in vivo* . (Hughes, et al., supra at S347). M-CSF, which has

previously been found to be essential for osteoclast formation.can suppress apoptosis, suggesting not only that maintenance of osteoclast populations, but also that formation of these multinucleated cells may be determined by apoptosis events. (Fuller, et al., *J. Bone Min. Res.* **8**, S384 (1993); Perkins, et al., *J. Bone Min. Res.* **8**, S390 (1993)). Local injections of IL-1 over the calvaria of mice once daily for 3 days induces intense and aggressive remodeling. (Wright, et al., *J. Bone Min. Res.* **9**, S174 (1994)). In these studies, 1% of osteoclasts were apoptotic 1 day after treatment, which increased 3 days later to 10%. A high percentage (95%) of these apoptotic osteoclasts were at the reversal site. This data suggests that Caspases are functionally very important in osteoclast apoptosis.

Therefore, one aspect of the present invention is the promotion of apoptosis in osteoclasts as a novel therapy for inhibiting resorption in diseases of excessive bone loss, such as osteoporosis, using compounds of Formula (I) as defined herein.

Apoptosis can been induced by low serum in highly differentiated rat osteoblast-like (Ros 17/2.8) cells (Ihbe, et al., (1994) *J. Bone Min. Res.* **9**, S167)). This was associated with a temporal loss of osteoblast phenotype, suggesting that maintenance of lineage specific gene expression and apoptosis are physiologically linked. Fetal rat calvaria derived osteoblasts grown *in vitro* undergo apoptosis and this is localized to areas of nodule formation as indicated by *in situ* end-labeling of fragmented DNA. (Lynch, et al., (1994) *J. Bone Min. Res.* **9**, S352). It has been shown that the immediate early genes c-fos and c-jun are expressed prior to apoptosis; c-fos and c-jun-Lac Z transgenic mice show constitutive expression of these transcription factors in very few tissues, one of which is bone (Smeyne, et al., (1992) *Neuron.* **8**, 13-23; and Morgan, J. (1993) Apoptotic Cell Death: Functions and Mechanisms. Cold Spring Harbor 13-15th October). Apoptosis was observed in these animals in the epiphyseal growth plate and chondrogenic zones as the petula ligament calcifies. Chondrogenic apoptosis has also been observed in PTHRP-less mice and these transgenics exhibit abnormal endochondral bone formation (Lee, et al., (1994) *J. Bone Min. Res.* **9**, S159). A very recent paper examined a human osteosarcoma cell line which undergoes spontaneous apoptosis. Using this cell line, LAP-4, but not ICE, could be detected and *in vitro* apoptosis could be blocked by inhibition or depletion of LAP-4 (Nicholson, et al., (1995) *Nature* **376**, 37-43). Thus, apoptosis may play a role in loss of osteoblasts and chondrocytes and inhibition of apoptosis could provide a mechanism to enhance bone formation.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to enhance bone formation using compounds of Formula (I) as defined herein.

Osteoarthritits (OA) is a degenerative disease characterized by progressive erosion of articular cartilage. Chondrocytes are the single cell-type found in articular cartilage and perturbations in metabolism of these cells may be involved in the pathogenesis of OA. Injury to cartilage initiates a specific reparative response which involves an increase in the production of proteoglycan and collagen in an attempt to reestablish normal matrix homeostasis. However, with the progress of the disease, the 3-dimensional collagen network is disrupted and cell death of chondrocytes occurs in OA lesions (Malemud, et al.: Regulation of chondrocytes in osteoarthritis. In: Adolphe, M. ed. Biological Regulation of Chondrocytes. Boca Raton:CRC Press, 1992, 295-319). It has been shown that in OA, chondrocytes adjacent to cartilage defects express high levels of bcl-2 (Erlacher, et al., (1995) *J. of Rheumatology*, 926-931). This represents an attempt to protect chondrocytes from apoptosis induced by the disease process.

Protection of chondrocytes during early degenerative changes in cartilage by inhibition of apoptosis may provide a novel therapeutic approach to this common disease. Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat osteoarthritis, using compounds of Formula (I) as defined herein.

Recent evidence shows that chronic, degenerative conditions of the liver are linked to hepatocellular apoptosis. These conditions include chemical-, infectious- and immune/inflammatory-induced hepatocellular degeneration. Apoptosis of liver cells has been observed in liver degenerative states induced by a variety of chemical agents, including acetaminophen (Ray, et a l.,(1993) *FASEB. J.* **7**, 453-463), cocaine (Cascales, et al., (1994) *Hepatology* **20**, 992-1001) and ethanol (Baroni, etal., (1994) *J. Hepatol.* **20**, 508-513). Infectious agents and their chemical components that have been shown to induce apoptosis include hepatitis ((Hiramatsu, et al., (1994) *Hepatology* **19**, 1354-1359; Mita, et al., (1994) *Biochem. Biophys. Res. Commun.* **204**, 468-474)), tumor necrosis factor and endotoxin . (Leist, et al., (1995) *J. Immunol.* **154**, 1307-1316; and Decker, K. (1993) *Gastroenterology* **28(S4)**, 20-25). Stimulation of immune / inflammatory responses by mechanisms such as allograft transplantation and hypoxia followed by reperfusion have been shown to induce apoptosis of hepatocytes (Krams, et al., (1995) *Transplant. Proc.* **27**,

466-467). Together, this evidence supports that hepatocellular apoptosis is central to degenerative liver diseases.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat degenerative liver diseases., using compounds of Formula (I) as defined herein.

Traumatic spinal injury often results in complete loss of voluntary motor and sensory function below the site of injury. Recent evidence shows that the long-term neurological deficits after spinal cord trauma may be due in part to widespread apoptosis of neurons and oligodendroglia in regions distant from and relatively unaffected by the initial injury. It has been found that certain upstream and downstream components of the Caspase-3 apoptotic pathway are activated after traumatic spinal cord injury in rats, and occur early in neurons in the injury site and hours to days later in the oligodendroglia adjacent to and distant from the injury site.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat spinal cord injury using compounds of Formula (I) as defined herein.

Apoptosis is recognized as a fundamental process within the immune system where cell death shapes the immune system and effects immune functions. Apoptosis also is implicated in viral diseases (e.g AIDS). Recent reports indicate that HIV infection may produce an excess of apoptosis, contributing to the loss of $CD4^+$ T cells. Of additional interest is the observation that APO-1/Fas shares sequence homology with HIV-1 gp120.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat viral diseases, using compounds of Formula (I) as defined herein.

Additional therapeutic directions and other indications in which inhibition of apoptotic cysteine proteases is of therapeutic utility, along with relevant citations in support of the involvement for apoptosis in each indication, are presented below in Table 1.

Table 1: Therapeutic Indications Related to Apoptosis

Indication	Citations
Ischemia / reperfusion	Barr et al., (1994) *BioTechnology* **12**, 487-493; Thompson, C. B. (1995) *Science* **267**, 1456-1462
Stroke	Barr et al supra; and Thompson, C., supra
Polycystic kidney disease	Barr et al., supra; and Mondain, et al., (1995) *ORL J. Otorhinolaryngol. Relat. Spec.* **57**, 28-32
Glomerulo-nephritis	Barr et al., supra
Osteoporosis	Lynch et al., (1994) *J. Bone Min. Res.* **9**, S352; Nicholson et al., (1995) *Nature* **376**, 37-43
Erythropoiesis / Aplastic anemia	Thompson, C., supra; Koury et al., (1990) *Science* **248**, 378-381
Chronic liver degeneration	Thompson, C., supra; Mountz et al., 1994) *Arthritis Rheum.* **37**, 1415-1420; Goldin et al., (1993) *Am. J. Pathol.* **171**, 73-76
T-cell death	Thompson, C., supra; Ameison et al., (1995) *Trends Cell Biol.* **5**, 27-32
Osteoarthritis – chondrocytes	Ishizaki et al., (1994) *J. Cell Biol.* **126**, 1069-1077; Blanco et al., (1995) *Am. J. Pathol.* **146**, 75-85
Male pattern baldness	Mondain et al., supra; Seiberg et al., (1995) *J. Invest. Dermatol.* **104**, 78-82; Tamada et al., (1994) *Br. J. Dermatol.* **131**, 521-524

Alzheimer's disease	Savill, J.,(1994) *Eur. J. Clin. Invest.* **24**, 715-723; Su et al., (1994) *Neuroreport* **5**, 2529-2533; Johnson, E., (1994) *Neurobiol. Aging* **15 Suppl. 2**, S187-S189
Parkinson's disease	Savill, J., supra; Thompson, C., supra
Type I diabetes	Barr et al., supra

The IL-1 and TNF inhibiting effects of compounds of the present invention are determined by the following *in vitro* assays:

Interleukin - 1 (IL-1)

Human peripheral blood monocytes are isolated and purified from either fresh blood preparations from volunteer donors, or from blood bank buffy coats, according to the procedure of Colotta *et al*, J Immunol, **132**, 936 (1984). These monocytes ($1x10^6$) are plated in 24-well plates at a concentration of 1-2 million/ml per well. The cells are allowed to adhere for 2 hours, after which time non-adherent cells are removed by gentle washing. Test compounds are then added to the cells for about 1hour before the addition of lipopolysaccharide (50 ng/ml), and the cultures are incubated at 37°C for an additional 24 hours. At the end of this period, culture super-natants are removed and clarified of cells and all debris. Culture supernatants are then immediately assayed for IL-1 biological activity, either by the method of Simon *et al.*, J. Immunol. Methods, **84**, 85, (1985) (based on ability of IL-1 to stimulate a Interleukin 2 producing cell line (EL-4) to secrete IL-2, in concert with A23187 ionophore) or the method of Lee *et al.*, J. ImmunoTherapy, **6** (1), 1-12 (1990) (ELISA assay).

Tumour Necrosis Factor (TNF):

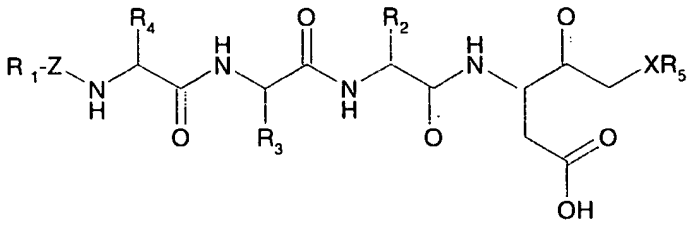
Human peripheral blood monocytes are isolated and purified from either blood bank buffy coats or platelet pheresis residues, according to the procedure of Colotta, R. *et al.*, J Immunol, **132**(2), 936 (1984). The monocytes are plated at a density of $1x10^6$ cells/ml medium/well in 24-well multi-dishes. The cells are allowed to adhere for 1 hour after which time the supernatant is aspirated and fresh medium (1ml, RPMI-1640, Whitaker Biomedical Products, Whitaker, CA) containing 1% fetal calf serum plus penicillin and streptomycin (10 units/ml) added. The cells are incubated for 45 minutes in the presence or absence of a test

compound at 1nM-10mM dose ranges (compounds are solubilized in dimethyl sulfoxide/ethanol, such that the final solvent concentration in the culture medium is 0.5% dimethyl sulfoxide/0.5% ethanol). Bacterial lipopoly-saccharide (*E. coli* 055:B5 [LPS] from Sigma Chemicals Co.) is then added (100 ng/ml in 10 ml phosphate buffered saline) and cultures incubated for 16-18 hours at 37°C in a 5% CO_2 incubator. At the end of the incubation period, culture supernatants are removed from the cells, centrifuged at 3000 rpm to remove cell debris. The supernatant is then assayed for TNF activity using either a radio-immuno or an ELISA assay, as described in WO 92/10190 and by Becker *et al.*, J Immunol, 1991, **147**, 4307.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the are can, using the preceding description, utilize the present invention to its fullest extent. Therefore the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

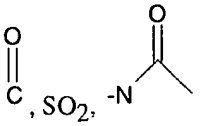
What is claimed is:

1. A compound of formula I



(I)

wherein

Z is  ;

R_1 is alkyl, alkylaryl or aryl;

R_2, R_3, and R_4 are selected from naturally occuring amino acids;

X is sulfur, oxygen or nitrogen and when X is sulfur, R_5 is C_{1-6} alkyl, alkylaryl or aryl

and when X is nitrogen R_5 is Y-R_6 and Y is SO_2, or  ;

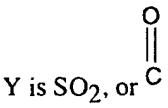
2. A compound according to claim 1 wherein:

R_1 is alkyl, alkylaryl or aryl;

R_2 is valine, isoleucine or threonine;

R_3 is glutamic acid or valine;

R_4 is aspartic acid;

R_5 is  and Y is SO_2, or ;

and X is Nitrogen.

3. A compound according to Claim 1 wherein X is nitrogen.

4. A compond according to Claim 1 wherein R_2 is valine, isoleucine or threonine.

5. A compound wherein R_3 is glutamic acid or valine.

6. A compound wherein R_4 is aspartic acid.

7. The compound according to claim 1 which is

Acetyl-aspartylglutamylvalylaspartyl benzoylaminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropionyl)aminomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylsulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (5-acetamido-3-methylthiophene)-2-sulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (5-benzoylaminomethylthiophene)-2-sulfonamidomethylketone
Acetyl-aspartylglutamylvalylaspartic acid (3-phenylpropyl)thiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid cyclohexylthiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid phenylthiomethylketone
Acetyl-aspartylglutamylvalylaspartic acid methylthiomethylketone

8. A pharmaceutical composition comprising a compound according to Claim 1 and a pharmaceutically acceptable carrier or diluent.

9. A method of blocking excess or inappropriate apoptosis in a mammal in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

10. The method according to Claim 7 wherein the excessive or inappropriate apoptosis occurs in Alzheimer disease.

11. The method according to Claim 7 wherein the excessive or inappropriate apoptosis occurs in viral infections.

12. The method according to Claim 7 wherein the excessive or inappropriate apoptosis occurs during infarction or reperfusion injury.

13. The method according to Claim 7 wherein the excessive or inappropriate apoptosis occurs during ischemia.

14. The method according to Claim 7 wherein the excessive or inappropriate apoptosis results in excessive bone loss.

15. The method according to Claim 7 wherein the excessive or inappropriate apoptosis results in the disease of osteoarthritis.

16. The method according to Claim 7 wherein the excessive or inappropriate apoptosis results in hepatocellular degeneration.

17. The method according to Claim 7 wherein the excessive or inappropriate apoptosis occurs during spinal cord injury.

18. A method for the treatment of diseases or disorders associated with excessive IL-1β convertase activity, in a mammal in need thereof, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

19. A method of blocking or decreasing the production of IL-1β and/or TNF, in a mammal in need of such treatment, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

20. A method for inhibiting the production of caspases 3, 7 and 8 in a mammal in need of such treatment, which method comprises administering to said mammal an effective amount of a compound of Formula I, or a pharmaceutically acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23271

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 5/08
US CL :530/331

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/331, 330; 514/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,055,451 A (KRANTZ et al) 08 October, 1991, see entire document	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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Date of the actual completion of the international search	Date of mailing of the international search report
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